*DEOXYRIBONUCLEIC ACID-DIRECTED SYNTHESIS OF RIBONUCLEIC ACID BY AN ENZYME FROM ESCHERICHIA COLI**

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Protein structure is under genetic control;[1-3] yet the precise mechanism by which DNA‡ influences the formation of specific amino acid sequences in proteins is unknown. Several years ago, it was discovered that infection of *Escherichia coli* with certain virulent bacteriophages induces the formation of an RNA fraction possessing both a high metabolic turnover rate and a base composition corresponding to the DNA of the infecting virus.[4-6] The existence of an analogous RNA component in noninfected cells has also been demonstrated; in this instance, however, the base composition of the RNA resembles that of the cellular DNA.[7, 8] These observations focused attention on the possible role of this type of RNA in protein synthesis, and some of the evidence consistent with this view has recently been summarized.[9]

Until recently there was no known enzymatic mechanism for a DNA-directed synthesis of RNA. Polynucleotide phosphorylase[10, 11] although it catalyzes the synthesis of polyribonucleotides, does not by itself provide a mechanism for the formation of RNA with a specific sequence of nucleotides. The one instance in which a unique sequence of nucleotides is produced involves the limited addition of nucleotides exclusively to the end of pre-existing polynucleotide chains.[12-14]

Our efforts were therefore directed toward examining alternate mechanisms for RNA synthesis, and in particular one in which DNA might dictate the nucleotide sequence of the RNA. In the present paper, we wish to report the isolation and some properties of an RNA polymerase from *E. coli* which, in the presence of DNA and the four naturally occurring ribonucleoside triphosphates, produces RNA with a base composition complementary to that of the DNA. Within the last year, several laboratories have reported similar findings with enzyme preparations from bacterial as well as from plant and animal sources.[15-24] In the following paper, the effect of enzymatically synthesized RNA on the rate and extent of amino acid incorporation into protein by *E. coli* ribosomes in the presence of a soluble protein fraction is described.

Experimental Procedure.—Materials: Unlabeled ribonucleoside di- and triphosphates were purchased from the Sigma Biochemical Corporation and the California Corporation for Biochemical Research. 8-C^{14}-labeled ATP was purchased from the Schwartz Biochemical Company; the other, uniformly labeled, C^{14} ribonucleoside triphosphates were prepared enzymatically from the corresponding monophosphate derivatives[25] isolated from the RNA of *Chromatium* grown on $C^{14}O_2$ as sole carbon source.[26] CTP labeled with P^{32} in the ester phosphate was obtained by enzymatic phosphorylation of CMP^{32} prepared according to Hurwitz.[27] The deoxyribonucleoside triphosphates were obtained by the procedure of Lehman *et al.*[25]

Calf thymus and salmon sperm DNA were isolated by the method of Kay *et al.*[28] DNA from *Aerobacter aerogenes, Mycobacterium phlei*, and bacteriophages T2, T5, T6 was prepared as described previously.[29] DNA from λ*dg* phage was prepared as reported elsewhere.[30] Unlabeled and P^{32} labeled DNA from *E. coli* were prepared as previously described.[31] d-AT and d-GC polymers were prepared according to Schachman *et al.*[32] and Radding *et al.*,[33] respectively. Transforming DNA from *Bacillus subtilis*[34] was a gift from E. W. Nester, and DNA from phage ØX

174 [49] was generously supplied by R. L. Sinsheimer. Double-stranded ØX 174 DNA was synthesized using *E. coli* DNA polymerase[25] with single-stranded ØX 174 DNA as primer.[35, 36] In this reaction, 2.7 times more DNA was synthesized than had been added as primer. RNA from tobacco mosaic virus was obtained from H. Fraenkel-Conrat, and ribosomal and amino acid-acceptor RNA were isolated from *E. coli* according to Ofengand *et al.*[26, 37] Nucleic acid concentrations are given as mμmoles of nucleotide phosphorus per ml.

Glass beads, "Superbrite 100," obtained from the Minnesota Mining and Manufacturing Company, were washed as previously described.[25] Streptomycin sulfate was a gift from Merck and Company, and protamine sulfate was purchased from Eli Lilly Company. DEAE-cellulose was purchased from Brown and Company. Crystalline pancreatic RNase and pancreatic DNase were products of the Worthington Biochemical Co.

Assays: The activities of *E. coli*-DNA polymerase,[25]-deoxyribonuclease[38] and -DNA diesterase,[31] were determined as previously described and ribonuclease activity was measured by the disappearance of amino acid-acceptor RNA activity.[26] Polynucleotide phosphorylase was measured by P_i^{32} exchange with ADP as reported by Littauer and Kornberg.[11] Protein was determined by the method of Lowry *et al.*[39]

The standard assay for RNA polymerase measures the conversion of either C^{14} or P^{32} from the labeled ribonucleoside triphosphates into an acid-insoluble form. Enzyme dilutions were made with a solution containing 0.01 *M* Tris buffer, pH 7.9, 0.01 *M* $MgCl_2$, 0.01 *M* β-mercaptoethanol, 5×10^{-5} *M* EDTA, and 1 mg per ml of crystalline bovine serum albumin. The reaction mixture (0.25 ml) contained: 10 μmoles of Tris buffer, pH 7.9, 0.25 μmole of $MnCl_2$, 1.0 μmole of $MgCl_2$, 100 mμmoles each of ATP, CTP, GTP, and UTP, 250 mμmoles of salmon sperm DNA, 3.0 μmoles of β-mercaptoethanol, and 10 to 80 units of enzyme. One of the nucleoside triphosphates was labeled with approximately 300 to 600 cpm per mμmole. After incubation at 37° for 10 min, the reaction mixture was chilled in ice, and 1.2 mg of serum albumin (0.03 ml) was added, followed by 3 ml of cold 3.5% perchloric acid (PCA). The precipitate was dispersed, centrifuged for 5 min at 15,000 × *g*, and washed twice with 3.0 ml portions of cold PCA. The residue was suspended in 0.5 ml of 2 *N* ammonium hydroxide, transferred to an aluminum planchet, and after drying, counted in a windowless gas-flow counter.

One unit of enzyme activity corresponds to an incorporation of 1 mμmole of CMP^{32} per hr under the conditions described above. The assay was proportional to the amount of enzyme added up to at least 80 units; thus 6.3, 12.5, and 25 μg of Fraction 4 enzyme incorporated 2.6, 5.1, and 10.0 mμmoles of CMP^{32}. The rate of the reaction remained constant for approximately 20 min, and then decreased after this time.

Since the radioactivity incorporated represents only one of the four nucleotides, the observed incorporation must be multiplied by a factor ranging from 3 to 5 for an estimate of the total amount of RNA synthesized. The exact factor depends on the composition of the DNA primer used.

Results.—Purification of RNA polymerase: (1) *Cells: E. coli* B was grown in continuous exponential phase culture[40] with a glucose–mineral salts medium.[41] Cells stored at −20° showed no loss of activity for over six months. The purification procedure and the results of a typical preparation are summarized in Table 1.

TABLE 1

PURIFICATION OF RNA POLYMERASE FROM *E. coli*

Fraction	Volume (ml)	Specific activity (units/mg)	Total activity (units)
1. Initial extract	260	40	370,000
2. Protamine eluate	37	1,600	205,000
3. Ammonium sulfate	5	2,500	200,000
4. Peak DEAE fraction	2	6,100	153,000

Unless noted otherwise, all operations were carried out at 4° and all centrifugations were at 30,000 × *g* for 15 min in an International HR-1 Centrifuge.

(2) *Extract:* Frozen cells (140 gm) were mixed in a Waring Blendor with 420 gm of glass beads and 150 ml of a solution (buffer A) containing 0.01 *M* Tris buffer, pH

7.9, 0.01 M $MgCl_2$, and 0.0001 M EDTA. After disruption of the cells at high speed for 15 min (maximum temperature 10°), a further 150 ml of buffer A was added and the glass beads were allowed to settle. The supernatant fluid was then decanted and the residue was washed with 75 ml of buffer A. The combined supernatant fluid and wash was centrifuged for 30 min and the resulting supernatant fluid collected (Fraction 1).

(3) *Streptomycin-protamine fractionation:* Fraction 1 was centrifuged in the Spinco Model L preparative ultracentrifuge for 4 hr at 30,000 rpm in the No. 30 rotor. The protein concentration in the supernatant fluid was adjusted to about 12 mg per ml with buffer A, and β-mercaptoethanol was added to a final concentration of 0.01 M. To 350 ml of the diluted supernatant solution was added 17.5 ml of a 10% (w/v) solution of Streptomycin sulfate with stirring. After 15 min, the solution was centrifuged, and to 350 ml of the supernatant fluid was added 14.0 ml of a 1% (w/v) solution of protamine sulfate. The precipitate, collected by centrifugation, was washed by suspension in 175 ml of buffer A containing 0.01 M β-mercaptoethanol. The washed precipitate was then suspended in 35 ml of buffer A containing 0.01 M mercaptoethanol and 0.10 M ammonium sulfate, centrifuged for 30 min, and the supernatant fluid was collected (Fraction 2).

(4) *Ammonium sulfate fractionation:* To 37 ml of Fraction 2 was added 15.8 ml of ammonium sulfate solution (saturated at 25° and adjusted to pH 7 with ammonium hydroxide). The mixture was stirred for 15 min, and the precipitate was removed by centrifugation. To the supernatant liquid was added an additional 16.2 ml of the saturated ammonium sulfate, and after 15 min the precipitate was collected by centrifugation for 30 min and dissolved in buffer B (0.002 M KPO_4, pH 8.4, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 0.0001 M EDTA) to a final volume of 5.0 ml (Fraction 3).

(5) *Adsorption and elution from DEAE-cellulose:* Fraction 3 was diluted to a protein concentration of about 3 mg per ml with buffer B and passed onto a DEAE-cellulose column (10 cm × 1 cm^2, washed with 150 ml of buffer B just prior to use) at a rate of about 0.5 ml per min. The column was washed with 10 ml of buffer B and then with enough of the same buffer containing 0.16 M KCl to reduce the absorbency of the effluent at 280 mμ to less than 0.05. The enzyme was eluted from the column with buffer B containing 0.23 M KCl. The activity appears within the first five ml of the latter eluant (Fraction 4).

(6) *Properties of the purified enzyme:* The specific activity of enzyme Fraction 4 was from 140 to 170 times greater than that of the initial extract. The purification as described here has been quite reproducible, with specific activities in the final fraction ranging from 5,500 to 6,100. The enzyme preparation (Fraction 4) has a ratio of absorbencies at 280 and 260 mμ of 1.5.

Fraction 4, stored at 0 to 2°, retains more than 90 per cent of its activity for up to two weeks and 40 to 60 per cent of the original activity after one month. Enzyme Fractions 1 through 3 are unstable, losing up to 30 per cent of their activity on overnight storage under a variety of conditions. Because of the marked instability of these earlier fractions, it is advisable to carry out the purification without stopping at intermediate stages.

(7) *Contaminating enzymatic activities:* Aliquots (100 μg) of Fraction 4 were assayed for contaminating enzymatic activities. This amount of enzyme cata-

lyzed an initial rate of incorporation of 2,000 mμmoles of nucleotide per hr. No detectable DNA polymerase was found (< 0.6 mμmole DNA per hr). DNase activity was barely detectable under conditions optimal for RNA polymerase. With either heated or unheated P^{32} DNA as substrate, no more than 0.13 mμmole of acid-soluble P^{32} was released during the course of a 30-min incubation. There was only slight RNase activity associated with Fraction 4. When 100 μg of the purified enzyme were incubated with 4 μmoles of purified acceptor RNA for 1 hr, there was no detectable inactivation of leucine-acceptor activity. Under similar conditions, 1 mg of enzyme produced a 30 per cent decrease in leucine-acceptor activity. With conditions optimal for RNA polymerase, sufficient polynucleotide phosphorylase activity was present to catalyze the exchange of 6.7 mμmoles of P_i^{32} into ADP per hour.

Requirements for the RNA polymerase reaction: With the purified enzyme, RNA synthesis was dependent on the addition of DNA, a divalent cation, and the four ribonucleoside triphosphates (Table 2). In a later section, we shall describe a

TABLE 2

REQUIREMENTS FOR RNA SYNTHESIS

Components	Incorporation of CMP^{32} (mμmoles)
Complete system	7.3
minus Mn^{++}	4.3
minus Mg^{++}	5.6
minus Mn^{++} and Mg^{++}	<0.03
minus DNA	<0.03
minus ATP, GTP, UTP	0.09
minus enzyme	<0.03

The standard system and assay procedure were used with 7.4 μg of Fraction 4 protein in each tube, except that $MgCl_2$ was omitted from the enzyme diluent.

TABLE 3

THE REQUIREMENT FOR RIBONUCLEOSIDE TRIPHOSPHATES IN RNA SYNTHESIS

Components	Incorporation of CMP^{32} (mμmoles)
Complete system	4.6
minus ATP	0.08
minus UTP	<0.03
minus GTP	<0.03
ATP, UTP, GTP replaced by dATP, dTTP, dGTP	0.05
ATP, UTP, GTP replaced by ADP, UDP, GDP	0.29

The standard system and assay procedure were used except that 250 mμmoles of calf thymus DNA were used as primer. 13 μg of Fraction 4 protein were used in each assay. 100 mμmoles of each nucleotide were added to each assay.

reaction in which ATP is converted to an acid-insoluble form in the absence of the other three triphosphates. Omission of β-mercaptoethanol from the reactiom mixture resulted in a 50 per cent loss in activity; however, dilution of the concentrated enzyme into solutions not containing a sulfhydryl compound resulted in as much as 90 per cent inactivation. The optimal pH for the reaction was between 7.8 to 8.2. At pH 6.1, 7.0, and 8.9 the activities were 13, 62, and 84 per cent, respectively, of the maximal value.

(1) *Nucleoside triphosphate specificity:* All of the ribonucleoside triphosphates are required for RNA synthesis (Table 3). The deoxyribonucleoside triphosphates do not function as substrates in the reaction, and the ribonucleoside diphosphates support synthesis only at a greatly reduced rate. The observed activity of the diphosphates may be due to the presence of small amounts of the nucleoside triphosphates in the diphosphate preparations or to the formation of the triphosphates through the action of nucleoside diphosphate kinase.

With CTP^{32} as the labeled substrate and salmon sperm DNA as primer, variation of the concentrations of all four ribonucleoside triphosphates as a group produced a variation in the rate of RNA synthesis. When the data were plotted according to Lineweaver and Burk,[42] a linear relationship was obtained from which

TABLE 4

THE EFFECT OF DIFFERENT NUCLEIC ACID PREPARATIONS ON THE RATE OF RNA SYNTHESIS BY RNA POLYMERASE

Source of primer	Incorporation of CMP^{32}*
DNA	
Salmon sperm	100
Calf thymus	43
E. coli	34
ØX 174	38
B. subtilis	27
λdg phage	25
T2 phage	45
T6 phage	30
T5 phage	74
RNA	
E. coli amino acid-acceptor	<0.5
E. coli ribosomal	<0.5
TMV	<0.5

* The incorporation value for salmon sperm DNA was 5.3 mμmoles and is set at 100 for comparison with the other primers.

Assay system and procedure as previously described, except that 100 mμmoles of each nucleic acid were used in place of the usual primer. 7.4 μg of Fraction 4 protein were used in each assay.

TABLE 5

THE EFFECT OF DENATURATION ON THE ABILITY OF DNA PREPARATIONS TO PRIME FOR RNA SYNTHESIS

DNA	Incorporation of CMP^{32} (mμmoles) Native	Heated
Calf thymus	2.7	2.3
Salmon sperm	6.1	2.5
T6 phage	1.9	0.8
E. coli	1.8	1.9

Assay procedure as described previously, except that the usual primer was replaced by 200 mμmoles of the DNA to be tested. 7.4 μg of Fraction 4 protein were used in each assay. The DNA samples were heated for 10 min at 95 to 99° in 0.05 *M* NaCl and rapidly cooled in an ice bath. The absorbencies of the heated DNA preparations were 30 to 40 per cent higher than those of the unheated preparations at 260 mμ.

it was calculated that the rate of synthesis was half maximal when the concentration of each of the triphosphates was 1.3×10^{-4} *M*. A similar value (1.4×10^{-4} *M*) was obtained using C^{14} ATP as a label and calf thymus DNA as primer.

(2) *The nature of the primer:* All DNA samples tested were active in promoting ribonucleotide incorporation, although the efficiency varied significantly (Table 4). Amino acid–acceptor RNA, ribosomal RNA from *E. coli*, and TMV RNA did not substitute for DNA. With the synthetic copolymer d-AT as primer, only AMP and UMP were incorporated, and only GMP and CMP were incorporated in the presence of d-GC polymer (Table 7). It should be noted, however, that GTP was incorporated to a considerably greater extent in the latter case. A qualitatively similar finding has been reported for the incorporation of dGMP and dCMP by DNA polymerase with d-GC as primer.[33]

Increasing the amount of DNA in an assay mixture over the range 0 to 200 mμmoles resulted in an increase in nucleotide incorporation. Further increases in the amount of DNA, up to 400 mμmoles, had no effect on the rate of RNA synthesis. A similar experiment using calf thymus DNA as primer gave a saturating value of 250 mμmoles.

The effect of disrupting the DNA double helix by heating[43] is shown in Table 5. It is seen that with several of the DNA preparations there is a significant decrease in the rate of CMP^{32} incorporation using the heated DNA while with others the effect is insignificant. The ability of single-stranded DNA to function as a primer for RNA synthesis is further emphasized by the activity of the single-stranded DNA from ØX 174 phage.

(3) *Metal ion requirements:* Optimal concentrations for Mn^{++} and Mg^{++} when added separately to the reaction mixture were 2×10^{-3} *M* and 8×10^{-3} *M*, respectively (Figure 1). Addition of Mg^{++} increased the rate at suboptimal levels of Mn^{++}; thus, the addition of 10^{-3} *M* Mn^{++} and 4×10^{-3} *M*

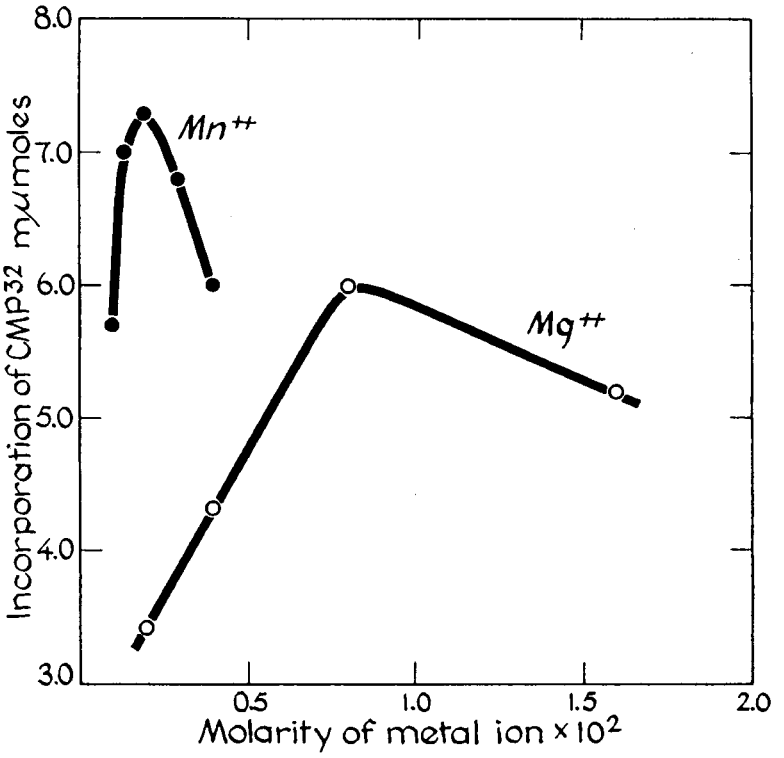

FIG. 1.—The Influence of Metal Ion Concentration on the Rate of CMP^{32} Incorporation. Standard assay conditions were used, except that the metal ion concentration was varied as shown. 7.4 μg of Fraction 4 enzyme were added to each assay.

O——O Mg^{++} alone added to the assay mixture

●——● Mn^{++} alone added to the assay mixture

Mg^{++} to the same reaction mixture gave a rate of incorporation equal to that found with the optimal concentration of Mn^{++} alone (2×10^{-3} *M*).

Characterization of the enzymatically synthesized RNA: (1) *Net synthesis of the RNA product:* With two times the level of the four ribonucleoside triphosphates and five to ten times the amount of enzyme used in the routine assay, the amount of RNA formed during an extended incubation exceeded the amount of DNA added to the reaction. We will designate this as "net synthesis." With most of the DNA preparations used, the amount of RNA formed was three to five times greater than the amount of DNA added, while with d-AT copolymer, up to 15 times as much of the corresponding AU polynucleotide was produced (Table 6). The rate of synthesis decreased after the first 20 min although further synthesis occurred up to two hr. Preliminary experiments indicate that this was not due to enzyme inactivation nor to destruction of the priming DNA, but other possibilities have not yet been investigated in detail.

(2) *Enzymatic and alkaline degradation of the product:* Exposure of the isolated "net synthesis" product to alkali converted > 98 per cent of the label to acid-soluble products which were electrophoretically identical with the 2′-(3′) nucleoside monophosphates. Treatment with pancreatic DNase or *E. coli* DNA diesterase[31] produced no significant liberation of labeled acid-soluble products.

TABLE 6

NET SYNTHESIS OF RNA

Source of DNA primer	Labeled nucleotide incorporated	Calculated amount of RNA formed* (mμmoles)	Ratio of RNA isolated to DNA added	Method of isolation
	CMP^{32}			
Calf thymus	81	200	2.0	A
φX 174 phage	90	510	5.1	A
T2 phage	78	360	3.6	A
T2 phage	72	410	4.1	B
	C^{14}-AMP			
T2 phage	150	460	4.6	C
T5 phage	152	500	5.0	C
d-AT copolymer	155	310	15.0	C

* The amount of RNA in the isolated product was calculated from the amount of label incorporated and the base ratio of the primer DNA.

Synthesis: Each tube contained in a final volume of 0.5 ml: 20 μmoles of Tris buffer, pH 7.85; 8 μmoles of $MgCl_2$; 400 mμmoles each of ATP, CTP, UTP, GTP; 6 μmoles of β-mercaptoethanol; 100 mμmoles of DNA; and 100 μg of Fraction 4 protein. When d-AT was used as primer, only 20 mμmoles of primer were added and CTP and GTP were omitted from the mixture. The incubation time was 3 hr at 37°.

Product isolation: A. The incubation mixture was heated for 10 min at 60° in 0.4 *M* NaCl, then dialyzed 36 hr against 0.2 *M* NaCl–0.01 *M* Tris, pH 7.85. B. The reaction mixture was extracted two times with phenol and the phenol fractions were washed two times with 0.4 *M* NaCl. The aqueous layers were pooled and dialyzed as in A. C. The product was precipitated from the incubation mixture with a solution containing 60 per cent ethanol and 0.5 *M* NaCl at 0°, washed once with the same solution, and dissolved in 1 ml of 0.2 *M* NaCl., then dialyzed as in A.

Treatment of 10 to 20 mμmoles of enzymatically prepared CMP^{32}-labeled RNA with 0.1 μg of pancreatic RNase for 1 hr liberated 75 to 94 per cent of the P^{32} label as acid-soluble products. The amount of acid-insoluble P^{32} remaining after RNase treatment varied with different DNA primers and different methods of product isolation. Using 10 times the amount of RNase did not appreciably alter the results. The significance of this RNase resistant fraction is presently unknown.

(3) *Nucleotide composition:* The nucleotide composition of the product was examined by two different methods. In the first method, four separate assays, each containing a different labeled nucleoside triphosphate, were performed with each DNA preparation, and the molar ratio in which the labeled nucleotides were incorporated was measured (Method A). The second method utilized electrophoretic separation[44] of the mononucleotides resulting from the alkaline degradation of a "net synthesis" product in which all of the nucleoside triphosphates were labeled with C^{14} (Method B). The distribution of the label among isolated nucleotides was therefore a measure of the composition of the newly synthesized RNA. The results (Table 7) indicate that the gross composition of the product at all stages of synthesis was complementary to that of the primer within the accuracy of the method. For double-stranded DNA, this complementary relationship becomes one of identity, since in the priming DNA adenine equals thymine and guanine equals cytosine. However, in the case of single-stranded ØX 174 DNA (Table 8), the composition is indeed complementary to that of the DNA, and in this instance the amounts of AMP and UMP incorporation and of GMP and CMP incorporation are not equal. Furthermore, when double-stranded ØX 174 DNA is used, the nucleotide composition of the resulting RNA is again identical to that of the DNA primer.

(4) *Sedimentation velocity of the isolated product:* The sedimentation velocity of the isolated RNA product was determined in the Spinco Model E analytical ultracentrifuge using ultraviolet optics. Values obtained (S_{20}) in 0.2 *M* NaCl–

TABLE 7

NUCLEOTIDE COMPOSITION OF THE RNA PRODUCT

DNA primer	Method of analysis	Nucleotide Composition (mμmoles) AMP	UMP	GMP	CMP	Primer* A + T / G + C	Product A + U / G + C	Product A + G / U + C
d-GC polymer	A	<0.03	<0.03	1.90	0.23	—	—	—
d-AT copolymer	B	21.7	20.0	—	—	—	—	—
d-AT copolymer	A	20.8	22.2	<0.03	<0.03	—	—	—
T2 phage	B	7.7	7.4	4.3	4.3	1.76	1.76	1.03
T5 phage	B	4.8	5.0	3.6	3.4	1.56	1.40	1.00
E. coli	A	1.8	1.9	1.9	2.0	1.01	0.95	0.95
M. phlei	A	3.7	4.0	7.9	8.5	0.48	0.47	0.93
A. aerogenes	A	1.8	1.7	2.3	2.2	0.80	0.78	1.05

* The values given for the ratio A + T/G + C in the priming DNA are those found by Josse *et al.*[30] except in the case of phage T5 DNA.[52]

Method A: For each DNA sample, four separate incubations were used, each containing a different C^{14}-labeled nucleotide. The amounts of DNA used in the various tests were as follows: 20 mμmoles of *M. phlei*, 50 mμmoles of *A. aerogenes*, 180 mμmoles of *E. coli*, 20 mμmoles of d-AT, 20 mμmoles of d-GC. 12.5 μg of Fraction 4 enzyme were used in each incubation; all other conditions were those given for a standard assay.

Method B: The synthesis of the C24-labeled RNA was carried out under the following conditions. The reaction mixture (0.5 ml) contained: 20 μmoles of Tris buffer, pH 7.85, 0.5 μmole of $MnCl_2$, 2 μmoles of $MgCl_2$, 6 μmoles of β-mercaptoethanol, 100 mμmoles each of C^{14}-ATP, C^{14}-UTP, C^{14}-GTP, C^{14}-CTP, 100 mμmoles of DNA, and 180 μg of Fraction 4 enzyme. Where d-AT primer was used, 20 mμmoles of primer were added and no CTP or GTP were added. After 180 min at 37°, the product was precipitated and washed with cold 3 per cent PCA and incubated in 0.3 *M* KOH for 18 hr at 37°. An aliquot to which carrier nucleotides had been added was subjected to paper electrophoresis at pH 3.5 in 0.05 *M* citrate buffer. The individual nucleotides which were visualized with a UV lamp were eluted in 0.01 *M* HCl and counted. Recovery of the C^{14}-label in the eluted fractions was >95 percent. 180 mμmoles, 140 mμmoles, and 200 mμmoles of polyribonucleotide were produced in the reactions primed with T2 DNA, T5 DNA, and d-AT, respectively.

TABLE 8

COMPARATIVE BEHAVIOR OF SINGLE- AND DOUBLE-STRANDED ØX 174 DNA AS PRIMER FOR RNA SYNTHESIS

State of DNA used as primer		Nucleotide Composition of RNA (per cent) AMP	UMP	GMP	CMP
Single-stranded	Predicted*	32.8	24.6	18.5	24.1
" "	Found by method A	32.0	24.1	19.5	24.3
" "	Found by method B	35.0	24.6	19.3	21.1
Double-stranded	Predicted*	28.7	28.7	21.3	21.3
" "	Found by method B	28.9	29.1	20.9	20.9

Method A: Conditions as given in Table 7. 32 mμmoles of single-stranded ØX 174 DNA were used in each incubation with 8 μg of Fraction 4 enzyme.

Method B: Conditions as given in Table 7. With single-stranded DNA as primer, 25 mμmoles of priming DNA were added, 71 mμmoles of RNA were produced in a 60 min incubation with 80 μg of Fraction 4 enzyme. For the double-stranded DNA, 26 mμmoles of priming DNA were added; 32 mμmoles of RNA were produced in a 60 min incubation with 40 μg of Fraction 4 enzyme.

* The predicted values were calculated on the assumption that the single-stranded ØX 174 DNA would yield RNA with a composition complementary to the composition reported by Sinsheimer.[49] Upon replication of ØX 174 DNA with DNA-polymerase it was assumed that the product (presumably double-stranded DNA) had a base composition which is the average of the composition of the original and of the newly synthesized strands.

That this is a reasonable assumption is shown by unpublished studies of M. Swartz, T. Trautner, and A. Kornberg. When ØX 174 DNA was used to prime limited (<30 per cent) or extensive (600 per cent) DNA synthesis, the composition of the newly formed DNA was:

	dAMP	TMP	dGMP	dCMP
Limited synthesis	31.0	24.1	20.1	24.5
Extensive synthesis	29.4	26.9	22.3	21.3

0.01 *M* Tris, pH 7.9, ranged from 6 to 7.5 for 2- to 15-fold "net synthesis" products prepared by phenol extraction or by salt-ethanol precipitation.

DNA-dependent formation of polyadenylic acid: As pointed out earlier, RNA synthesis, as measured by the incorporation of either labeled CTP, UTP, or GTP did not occur in the absence of the other three nucleoside triphosphates or, in fact, in the absence of any one of the nucleoside triphosphates. It was therefore surprising to find that purified fractions of RNA polymerase catalyze the conversion

of C^{14}-ATP to an acid-insoluble form in the absence of the other three ribonucleoside triphosphates. The ratio of the activities

$$\frac{\text{AMP incorporated in the absence of UTP, CTP, GTP}}{\text{AMP incorporated in the presence of UTP, CTP, GTP}}$$

increased from 0.5 to 10 as purification of the enzyme progressed.

(1) *Requirements for polyadenylic acid formation:* Polyadenylic acid formation from ATP occurred only in the presence of DNA, a divalent cation, and the purified enzyme (Table 9). Note that addition of unlabeled ADP produces only a small dilution of the incorporation of label from C^{14}-ATP. The rate of incorporation was directly proportional to the amount of enzyme added; 1.8, 3.6, and 7.2 μg of Fraction 4 enzyme catalyzed the incorporation of 4.0, 8.2, and 17.5 mμmoles of C^{14}-AMP in a standard 10 min assay. The rate of incorporation remained constant up to over 75 per cent utilization of the added ATP.

TABLE 9

REQUIREMENTS FOR POLYADENYLIC ACID FORMATION

System	Incorporation of AMP (mμmoles)
Complete (with ATP as the only nucleoside triphosphate)	9.9
minus DNA	<0.03
minus Mn^{++}	2.5
minus Mg^{++}	8.7
plus RNase	6.9
plus DNase	0.3
plus ADP	7.6

The reaction mixture contained, in a final volume of 0.25 ml: 10 μmoles of Tris buffer, pH 7.85; 0.5 μmole of $MnCl_2$; 2 μmoles of $MgCl_2$; 3 μmoles of β-mercaptoethanol; 100 mμmoles of C^{14}-ATP; 280 mμmoles of calf thymus DNA; and 3 μg of Fraction 4 RNA polymerase. Where indicated, 25 μg of pancreatic RNase, 25 μg of pancreatic DNase, and 100 mμmoles of ADP were added. The incubation time was 10 min at 37°.

TABLE 10

INCORPORATION OF SINGLE NUCLEOTIDES BY RNA POLYMERASE

Nucleotide added	Nucleotide incorporation (mμmoles)
C^{14}-ATP	23
C^{14}-UTP	0.90
C^{14}-GTP	0.09
CTP^{32}	0.07

The conditions were the same as those described in Table 9, except that ATP was replaced where indicated by an equal amount of each of the other nucleoside triphosphates. 6 μg of Fraction 4 enzyme were added.

There was no incorporation of CMP or GMP when the corresponding nucleoside triphosphates were added singly to the reaction, although UMP incorporation occurred to a small, but significant, extent (Table 10).

(2) *The DNA requirement for polyadenylic acid formation:* The ability of various nucleic acid preparations to support polyadenylic acid synthesis is shown in Table 11. Note that neither RNA nor polyadenylic acid itself replaced the DNA requirement. To test whether DNA might be necessary only to initiate polyadenylic acid synthesis, an experiment was performed in which the priming DNA was destroyed after some polyadenylic acid formation had already occurred. It can be seen that destruction of the DNA by DNase blocked further synthesis of the polyadenylic acid (Table 12). This implies that the DNA is required not only for the initiation of polyadenylic acid synthesis, but also for the continued formation of the polynucleotide.

(3) *The effect of the other ribonucleoside triphosphates on polyadenylic acid formation:* The addition of the other ribonucleoside triphosphates resulted in an inhibition of the rate of C^{14} AMP incorporation (Table 13). It can be seen, for

TABLE 11

PRIMING EFFICIENCY OF VARIOUS NUCLEIC ACID PREPARATIONS FOR POLYADENYLIC ACID FORMATION

Primer	AMP incorporation (mμmoles)
Calf thymus DNA	10
Salmon sperm DNA	7.7
T2 phage DNA	4.9
d-AT copolymer	<0.03
Amino acid-acceptor RNA	0.35
Polyadenylic acid	0.07

The conditions of the incubation were as described in Table 9, except that the following amounts of nucleic acid were added: 300 mμmoles of salmon sperm DNA; 100 mμmoles of T2 phage DNA; 12 mμmoles of d-AT; 110 mμmoles of amino acid–acceptor RNA and 5 mμmoles of polyadenylic acid. 3 μg of Fraction 4 enzyme were added.

TABLE 12

THE EFFECT OF DEOXYRIBONUCLEASE ADDITION DURING POLYADENYLIC ACID SYNTHESIS

Tube	Treatment	AMP incorporation (mμmoles)
1	5-min incubation	7.1
2	10-min incubation	16.2
3	10-min incubation	6.9

The reaction mixtures were as described in Table 9, except that 6 μg of Fraction 4 was used. Tube 1 was incubated for 5 min, heated for 3 min at 100°, and then assayed as usual. Tube 2 was incubated for 10 min before assaying. Tube 3 was incubated for 5 min and heated as in the case of tube 1; 25 μg of pancreatic DNase were then added and the mixture incubated for an additional 5 min. At this time, 6 μg of fresh RNA polymerase were added and a third 5-min incubation was allowed.

TABLE 13

EFFECT OF THE OTHER RIBONUCLEOSIDE TRIPHOSPHATES ON POLYADENYLIC ACID FORMATION

Component	AMP incorporation (mμmoles)
Complete system	26
plus CTP	6.0
plus UTP	5.2
plus GTP	2.0
plus CTP, UTP	1.3
plus CTP, GTP	0.6
plus UTP, GTP	0.5
plus UTP, GTP, CTP	2.2

Complete system as in Table 9, except that 6 μg of Fraction 4 protein were added. Where indicated, 100 mμmoles of each nucleoside triphosphate were added.

example, that in the presence of any two of the other triphosphates the amount of polyadenylic acid formed is less than 5 per cent that of the control in which only ATP was added. As has been previously shown, in the presence of all four triphosphates, AMP is incorporated into a product having a base composition determined by the DNA primer, and hence under these conditions polyadenylic acid synthesis does not appear to occur.

(4) *Characterization of the polyadenylic acid product:* Preliminary characterization of the product is consistent with its identity as a polyadenylic acid. The addition of pancreatic RNase to the assay system lowered the rate of incorporation only slightly (about 30%). Treatment with 0.5 *M* KOH for 18 hr at 37° converted the product to an acid-soluble form. Of the C^{14} in the hydrolysate, 97 per cent was associated with 2′-(3′) AMP on paper chromatography[44] and paper electrophoresis,[45] less than 1.5 per cent with adenosine, and less than 1.5 per cent was found in a region corresponding to adenosine 3′-5′ diphosphate. This implies that the minimum chain length of the polyadenylate is in the order of 60 to 70 nucleotide residues.

Discussion.—There is a striking similarity between the reactions catalyzed by the RNA polymerase described here and *E. coli* DNA polymerase.[25] Both use only the nucleoside triphosphates as nucleotidyl donors, and both display absolute requirements for a divalent cation and a DNA primer for polynucleotide synthesis.§ In both cases, some ambiguity exists as to the relative efficiency of single as compared to double-stranded DNA for priming of polynucleotide synthesis. In each

reaction, both forms of DNA are active as primers, but a meaningful comparison between the two with regard to the mechanism of priming must await a more detailed physical and chemical characterization of the different DNA preparations, and further purification of the enzymes involved.

The product formed by RNA polymerase, as in the analogous case of DNA polymerase[29] has a base composition which, within experimental error, is complementary to that of the priming DNA. This finding, which is in agreement with the results obtained by others[16, 17, 21], supports the view that the nucleotide sequences in the DNA direct the order of nucleotides in the enzymatically synthesized RNA. A more critical test of this hypothesis involves a comparison of the nucleotide sequence of the priming DNA and the newly synthesized RNA. In this regard, Furth *et al.*[19] have shown that the repeating sequence of dAMP and dTMP in d-AT copolymer is faithfully replicated by the RNA polymerase in the form of an alternating AMP and UMP sequence. More recently Weiss and Nakamoto[46] have shown that RNA synthesized with an RNA polymerase from *M. lysodeikticus* contains the same frequencies of dinucleotide pairs as occur in the DNA primer. Additional experiments[47] which demonstrate the formation of a DNA-RNA complex after heating and slow-cooling[48] suggest that the homology of nucleotide sequences may occur over relatively long regions.

Does RNA polymerase copy the sequence of only one or both strands of DNA? This question is relevant not only to an understanding of the enzymatic copying mechanism, but also to any speculations as to the mechanism of information transfer from DNA to RNA. The fact that with double-stranded DNA primers the base composition of the newly made RNA is essentially identical to the over-all composition of both strands of the DNA already suggests that each strand can function equally well. An alternative hypothesis is to suppose that only one strand can be copied, and that the "primer" strand has, in the case of every DNA studied, a base composition identical to the average composition of both strands. Using the double-stranded form of ØX 174 DNA[35, 36] in which it is known that the base compositions of the two strands differ,[49] it is possible to test this question directly. The results show that both strands of the duplex serve to direct the composition of the RNA product.

This result still leaves open the question of whether both strands are copied in one replication cycle or whether only one strand is copied at a time and the choice between strands is random. When considering the relevance of this finding to information transfer, one must bear in mind that the existence of artificially produced ends in an isolated DNA preparation may allow RNA formation to proceed from both ends of the double strand. This, however, may not occur with the DNA as it exists in the genome; that is, *in vivo* some structural feature in the chromosomal DNA may cause RNA synthesis to proceed in a unidirectional manner and therefore copy the sequence of only one of the two strands.

The formation of DNA-RNA complexes has been described by several groups of workers,[48, 50, 51] although only limited information is available concerning their chemical structure and their metabolic and chemical stability. The fact that in the enzymatic reaction net synthesis of RNA occurs argues against the formation of a stable, stoichiometric complex of RNA and DNA. A further argument against the formation of such a complex is the finding that most of the DNA re-

maining at the end of the reaction appears to be identical to the DNA added, and no component containing both DNA and newly synthesized RNA was detectable on CsCl gradient centrifugation.[47] Whether some transient complex is formed as an intermediate is somewhat more difficult to assess.

The formation of polyadenylic acid in a DNA-dependent reaction is significant in view of the fact that none of the other ribonucleoside triphosphates, taken singly or even in groups of three, are utilized to any appreciable extent for polynucleotide synthesis. An exception to this is, of course, the situation where the DNA dictates the incorporation of only one or two nucleotides (e.g., with poly dT,[18] d-AT,[19] or d-GC).

Three possibilities which could account for polyadenylic acid synthesis are that it results from (*a*) a special feature of RNA polymerase itself, (*b*) a separate polyadenylic acid polymerase, or (*c*) polynucleotide phosphorylase. The last possibility is least likely because of the absolute requirement for DNA in the initiation and continuation of synthesis, the failure of ADP to give a significant dilution of the incorporation from ATP, the low amounts of polynucleotide phosphorylase activity found in the enzyme preparation as measured by P_i^{32} exchange, the inability of Mg^{++} alone to support maximal rates of synthesis, the lack of polymerization of the other nucleoside triphosphates, and the marked inhibition of polyadenylic acid synthesis by any one or all four of the triphosphates The question of whether polyadenylic acid synthesis is catalyzed by RNA polymerase or by another enzyme cannot be resolved at the present time.

With regard to the mechanism of the DNA-dependent polyadenylic acid formation, two aspects deserve specific comment. The first concerns the mechanism of the inhibition of polyadenylic acid synthesis by any one or all of the other triphosphates. It should be recalled that polyadenylic acid synthesis does not occur in the presence of all four ribonucleoside triphosphates ($< 3\%$), since under these conditions the base composition of the newly synthesized RNA is very close to that predicted by the composition of the DNA primers. The second notable feature of the reaction is its complete dependence on DNA, and the failure of d-AT to prime polyadenylic acid synthesis. One way to account for these findings is to assume that a sequence of thymidylate residues in the DNA, which does not occur in d-AT, can prime the formation of a corresponding run of AMP residues and, by subsequent "slippage" of one chain along the other, lead to a DNA-dependent elongation of the polyadenylic acid chain. The introduction of any other nucleotide into the growing chain might block or inhibit the sliding process and thereby terminate the growing polyadenylic acid chain.

Summary.—An RNA polymerase has been isolated from *E. coli* which in the presence of the four ribonucleoside triphosphates, a divalent metal ion, and DNA synthesizes RNA with a base composition complementary to that of the priming DNA. Both strands of DNA can prime new RNA synthesis. Thus, while single-stranded ØX 174 DNA yields RNA with a base composition complementary to that of the single-stranded form, double-stranded ØX 174 DNA (synthesized with DNA polymerase) primes the synthesis of RNA with a base composition virtually the same as that in both strands of the DNA. A novel feature of the RNA polymerase preparations is their ability to catalyze a DNA-dependent formation of polyadenylic acid in the presence of ATP alone. Neither UTP, GTP, nor CTP yields corre-

sponding homopolymers; the DNA-dependent formation of polyadenylic acid is virtually completely inhibited by the presence of the other nucleoside triphosphates.

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‡ The abbreviations used in this paper are: RNA and DNA for ribo- and deoxyribonucleic acid, respectively; poly dT for polydeoxythymidylate; d-AT for the deoxyadenylate-thymidylate copolymer; d-GC for the deoxyguanylate-deoxycytidylate polymer: AMP, ADP and ATP for adenosine-5′-mono-, di-, and triphosphates, respectively. A similar notation is used for the cytidine (C), guanosine (G), and uridine (U) derivatives and their deoxy analogues (dA, dC, dG, dT). P_i is used for inorganic orthophosphate, TMV for tobacco mosaic virus, and DNase and RNase for deoxyribo- and ribonuclease activities, respectively.

§ Under certain conditions DNA polymerase preparations will, in the absence of DNA, produce d-AT or d-GC depending upon the nature of the substrates present.[32, 33]

[1] Ingram, V. M. and J. A. Hunt, *Nature* **178**, 792 (1956).

[2] Yanofsky, C. and P. St. Lawrence, *Ann. Rev. Microbiol.*, **14**, 311 (1960).

[3] Fincham, J. R. S., *Ann. Rev. Biochem.*, **28**, 343 (1959).

[4] Volkin, E. and L. Astrachan, *Virology*, **2**, 149 (1956).

[5] Volkin, E., these PROCEEDINGS, **46**, 1336 (1960).

[6] Nomura, M., B. D. Hall and S. Spiegelman, *J. Mol. Biol.*, **2**, 306 (1960).

[7] Yčas, M. and W. S. Vincent, these PROCEEDINGS, **46**, 804 (1960).

[8] Gros, F., W. Gilbert, H. Hiatt, P. F. Spahr, and J. D. Watson, Cold Spring Harbor Symposia on Quantitative Biology, vol. 21, in press.

[9] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).

[10] Ochoa, S. and L. Heppel, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1957), p. 615.

[11] Littauer, U. Z. and A. Kornberg, *J. Biol. Chem.*, **226**, 1077 (1957).

[12] Hecht, L. I., M. L. Stephenson, and P. C. Zamecnik, these PROCEEDINGS, **45**, 505 (1959).

[13] Canallakis, E. S. and E. Herbert, these PROCEEDINGS, **46**, 170 (1960).

[14] Preiss, J., M. Dieckmann, and P. Berg, *J. Biol. Chem.*, **236**, 1749 (1961).

[15] Weiss, S. B., these PROCEEDINGS, **46**, 1020 (1960).

[16] Weiss, S. B. and T. Nakamoto, *J. Biol. Chem.*, PC 18 (1961).

[17] Hurwitz, J., Bresler, A. and R. Diringer, *Biochem. Biophys. Res. Comm.*, **3**, 15 (1960).

[18] Furth, J. J., J. Hurwitz, and M. Goldmann, *Biochem. Biophys. Res. Comm.*, **4**, 362 (1961).

[19] *Ibid.*, **4**, 431 (1961).

[20] Stevens, A., *Biochem. Biophys. Res. Comm.*, **3**, 92 (1960).

[21] Stevens, A., *J. Biol. Chem.*, **236**, PC 43 (1961).

[22] Ochoa, S., D. P. Burma, H. Kröger, and J. D. Weill, these PROCEEDINGS, **47**, 670 (1961).

[23] Burma, D. P., H. Kröger, S. Ochoa, R. C. Warner, and J. D. Weill, these PROCEEDINGS, **47**, 749 (1961).

[24] Huang, R. C., N. Maheshwari, and J. Bonner, *Biochem. Biophys. Res. Comm.*, **3**, 689 (1960).

[25] Lehman, I. R., M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.*, **233**, 163 (1958).

[26] Ofengand, E. J., Ph.D. Thesis, Washington University, St. Louis, Missouri (1959).

[27] Hurwitz, J., *J. Biol. Chem.*, **234**, 2351 (1959).

[28] Kay, E. R. M., N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.*, **74**, 1724 (1952).

[29] Josse, J., A. D. Kaiser, and A. Kornberg, *J. Biol. Chem.*, **236**, 864 (1961).

[30] Kaiser, A. D., and D. S. Hogness, *J. Mol. Biol.*, **2**, 392 (1960).

[31] Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).

[32] Schachman, H. K., J. Adler, C. M. Radding, I. R. Lehman, and A. Kornberg, *J. Biol. Chem.*, **235**, 3243 (1960).

[33] Radding, C. M., J. Josse, and A. Kornberg, unpublished results.

[34] Nester, E. W., and J. Lederberg, these PROCEEDINGS, **47**, 56 (1961).

[35] Lehman, I. R., *Ann. N. Y. Acad. Sci.*, **81–3**, 745 (1959).

[36] Lehman, I. R., R. L. Sinsheimer, and A. Kornberg, unpublished results.

[37] Ofengand, E. J., M. Dieckmann, and P. Berg, *J. Biol. Chem.*, **236**, 1741 (1961).

[38] Lehman, I. R., G. G. Roussos, and A. Pratt, *J. Biol. Chem.*, in press.
[39] Lowry, O., J. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[40] Monod, J., *Ann. Inst. Pasteur*, **79**, 390 (1950).
[41] Wiesmeyer, H., and M. Cohn, *Biochim. Biophys. Acta*, **39**, 417 (1960).
[42] Lineweaver, H., and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
[43] Doty, P., these PROCEEDINGS, **42**, 791 (1956).
[44] Markham, R. and J. P. Smith, *Biochem. J.*, **52**, 552 (1952).
[45] Magasanik, B., E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).
[46] Weiss, S. B., and T. Nakamoto, these PROCEEDINGS, **47**, 1400 (1961).
[47] Geiduschek, E. P., T. Nakamoto, and S. B. Weiss, these PROCEEDINGS, **47**, 1405 (1961).
[48] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47**, 137 (1961).
[49] Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 43 (1959).
[50] Rich, A., these PROCEEDINGS, **46**, 1044 (1960).
[51] Schildkraut, C. L., J. Marmur, J. R. Fresco, and P. Doty, *J. Biol. Chem.*, **236**, PC 2 (1961).
[52] Wyatt, G. R., and S. S. Cohen, *Biochem. J.*, **55**, 774 (1953).